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A multi-criteria analysis approach for ranking and selection of microorganisms for the production of oils for biodiesel production

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ABSTRACT

Oleaginous microorganisms have potential to be used to produce oils as alternative feedstock for biodiesel production. Microalgae (*Chlorella protothecoides* and *Chlorella zofingiensis*), yeasts (*Cryptococcus albidus* and *Rhodotorula mucilaginosa*), and fungi (*Aspergillus oryzae* and *Mucor plumbeus*) were investigated for their ability to produce oil from glucose, xylose and glycerol. Multi-criteria analysis (MCA) using analytic hierarchy process (AHP) and preference ranking organization method for the enrichment of evaluations (PROMETHEE) with graphical analysis for interactive aid (GAIA), was used to rank and select the preferred microorganisms for oil production for biodiesel application. This was based on a number of criteria *viz*., oil concentration, content, production rate and yield, substrate consumption rate, fatty acids composition, biomass harvesting and nutrient costs. PROMETHEE selected *A. oryzae*, *M. plumbeus* and *R. mucilaginosa* as the most prospective species for oil production. However, further analysis by GAIA Webs identified *A. oryzae* and *M. plumbeus* as the best performing microorganisms.

Keywords: Microbial oil, Multi-criteria analysis, Glucose, Xylose, Glycerol

1. Introduction

With increasing population and development, global petroleum demand is predicted to increase by up to 40% by 2025 (Subramaniam et al., 2010). In this regard, renewable energy technologies can contribute to meet a portion of the increase, while addressing some of the major concerns with greenhouse gas emissions from the continued use of fossil fuels. Biodiesel, commonly produced from vegetable oils, is a renewable transportation fuel that has received widespread acceptance and uptake. However, the use of edible oils for biodiesel production will contribute to increase food prices because of growing demand for use in both fuels and food products (Subramaniam et al., 2010). Microorganisms have the potential to be used to produce oils as alternative feedstock for biodiesel production and reduce the amount of edible oils used for this purpose. In addition, microbial oils have the potential to be utilised for the production of other products depending on the fatty acid profiles of the oil produced. These products include cocoa butter substitutes and health products such as γ-linolenic acid (GLA), arachidonic acid (ARA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Huang et al., 2013; Ratledge, 2004).

Microorganisms from certain species of microalgae, yeasts, fungi and bacteria are able to accumulate lipids (*i.e.*, oils) at more than 20% dry weight of biomass (Ratledge, 2004). Lipid production is typically optimised under nitrogen-limiting conditions with carbon substrates in excess (Evans & Ratledge, 1984). Lipid accumulation in oleaginous microorganisms is due to the presence of ATP-citrate lyase (ATP-CL) (Ratledge, 2004). ATP-CL catalyses the formation of acetyl-CoA, which is then used in fatty acid biosynthesis (Ratledge, 2004).

Lignocellulosic biomasses, from agricultural crop residues such as sugarcane bagasse and palm oil empty fruit bunch, are rich in carbohydrates. These carbohydrates, which can be hydrolysed to fermentable sugars such as glucose and xylose, provide low cost carbon source for microbial oil production as lignocellulosic biomasses are renewable and abundant (Huang et al., 2013). So, it is of interest to study the production of oils from lignocellulose hydrolysates using selected microorganisms. The first step, reported here is on the use of glucose and xylose as model substrates.

Certain species of microalgae have been shown to produce oil through phototrophic or heterotrophic cultivation (Chen et al., 2011). In particular, *Chlorella* species are capable of producing oil with high yields such as *Chlorella vulgaris* and *Chlorella protothecoides* that have been widely studied for heterotrophic cultivation, principally with the use of glucose or fructose-based substrates (Chen et al., 2011; Miao & Wu, 2006). There are only limited studies on the growth of *Chlorella* on pentoses such as xylose probably because microalgae generally do not use pentoses (Hawkins, 1999). In addition, the use of microalgae for heterotrophic cultivation may be prone to contamination in the presence of high sugar concentrations in the growth media due to their low growth rates (Chen et al., 2011).

Besides microalgae, yeasts and fungi are other microorganisms used for lipid production from various carbon sources. There are several species of yeasts that are known for their oil accumulating capability growing on various carbon substrates such as *Yarrowia lipolytica*, *Rhodosporidium toruloides*, *Lipomyces starkeyi*, *Trichosporon fermentans*, *Trichosporon pullulan*, *Rhodotorula glutinis* and *Cryptococcus curvatus* (Li et al., 2010; Meng et al., 2009). Oil production by yeast cultivation is promising as yeasts often exhibit high growth rates with low nutrient requirements, and certain

species have been shown to have a high oil accumulation capability with fatty acid composition comparable to plant oils (Kitcha & Cheirsilp, 2011; Meng et al., 2009; Saenge et al., 2011).

Filamentous fungal species such as *Mortierella isabellina* and *Cunninghamella echinulata* are reported to accumulate high oil contents from several carbon substrates (Chatzifragkou et al., 2011; Zheng et al., 2012). Similar to the oil extracted from microalgae and yeasts, these fungal oils can also be used as a feedstock for biodiesel production (Chatzifragkou et al., 2011; Zheng et al., 2012).

It is concluded, therefore, that each class and species of microorganisms exhibits advantages and disadvantages for industrial oil production. In addition, there are many criteria that influence the commercial potential of a microorganism. Most studies to date have used oil content and oil concentration as the selection criteria, and have paid little attention to other significant criteria that contribute to an economically viable oil production process. Key criteria likely to be important in microorganisms ranking and selection for oil and biodiesel production include:

1. Oil concentration (g/L);

2. Oil content (g/g microbial biomass);

3. Oil production rate (productivity; g/L/day);

4. Oil yield (oil concentration per unit substrate consumed; g/g consumed substrate);

5. Substrate consumption rate (g/L/h);

6. Fatty acids profile (%, *w*/*w*);

7. Biomass harvesting cost ($/L); and

8. Nutrient cost ($/L).

It is likely that no single microorganism will exhibit the optimum performance for each of these criteria and hence selection of prospective microorganisms will require a compromise decision in order to select the best overall performance. In lignocellulosic hydrolysates, glucose and xylose are typically the major carbohydrate monomers. Glycerol is produced in large quantities as by-product from biodiesel production and represents an additional potential feedstock for microbial oil production in integrated microbial oil and biodiesel production facilities. The performance of a microorganism may vary across each of these substrates. Other factor that affects performance of a microorganism is inhibitory effect of degradation products from the pretreatment process (*e.g.*, furfural from pentose and 5-hydroxymethylfurfural from hexose) (Yu et al., 2011). This factor however is considered to be secondary to the key criteria listed, as it depends on type of hydrolysate used. The inhibitors are generally prevalent in hydrolysates from the liquid fraction of pretreated lignocellulosic materials, but it is negligible in enzymatic hydrolysates from washed solid residues. The effects of inhibitors to microbial growth may be reduced by detoxifying hydrolysates, such as through overliming process (Yu et al., 2011).

Further complicating the selection decision for lignocellulosic hydrolysates is that there is no standard lignocellulose or lignocellulosic hydrolysate and composition varies with biomass type, age of plant, climatic conditions during growth, pre-processing and pretreatment technology and severity. As a result, most screening studies using lignocellulosic hydrolysates focus on a specific biomass and pretreatment

technology. It is likely, therefore, that the results of screening studies will be specific to the factors used in the selection.

At the present time, there is no reported methodology for systematic evaluation of prospective microorganisms that accounts for the diversity of criteria needed for an economically viable oil production process. So, it is proposed that multi-criteria analysis (MCA) methods can be used to provide flexible analytical tools to aid complex decision making in the ranking and selection of alternatives (*i.e.*, microorganisms) (Herva & Roca, 2013). Preference Ranking Organization Method for the Enrichment of Evaluations (PROMETHEE) is a computer-based multi-criteria decision aid methodology to rank alternative solutions to a complex problem. PROMETHEE uses outranking techniques for alternatives based on the weightings of selected preferences to determine positive and negative preference flows (Behzadian et al., 2010). The PROMETHEE I Partial Ranking consists of positive preference flows (*Phi*+) which measures the extent to which an alternative outranks all others; and negative preference flows (*Phi*-) which measures the extent to which an alternative is outranked by others (Brans & Mareschal, 2005). The PROMETHEE II Complete Ranking (*Phi*) is a calculation of the net preference flow that shows the balance between the positive and negative outranking flows (Brans & Mareschal, 2005). Graphical Analysis for Interactive Aid (GAIA) is a visual aid tool used with PROMETHEE that enables visualisation and graphical representation of the analysis.

In a study assessing algae from nine different species for biodiesel production, PROMETHEE-GAIA was used as the tool for multi-criteria decision making (Islam et al., 2013). PROMETHEE-GAIA was used for systematic analysis and graphical representation of the most preferred and the least preferred species, based on multiple

physical and chemical properties of fuel (*e.g.*, oil concentration and cetane number) as the selection criteria. However, in this study, equal weight was applied to each criterion in PROMETHEE. The assessment from this model is not accurate for any particular scenario that has fuel properties that are more important than others. The best species selected should reflect the best quality in the most desired criterion or fuel property, and a compromise quality in the least desired criterion will not give major effect to the preference results.

It is essential to use structured technique for determining weights for complex MCA. This is because there is no guidelines in PROMETHEE II for weight determination, but decision makers are assumed to be able to assign appropriate weight to each criterion (Macharis et al., 2004). An environmental evaluation study of municipal solid waste options used the combination of Analytic Hierarchy Process (AHP) and PROMETHEE for MCA (Herva & Roca, 2013). AHP is another complex decision making support technique that provides a structured process for the identification of hierarchies of goals, criteria and alternatives for evaluation (Macharis et al., 2004). AHP is widely used for developing weightings of criteria. The combination of PROMETHEE and AHP for MCA was proposed by Macharis et al. (2004). In the environmental evaluation study by Herva & Roca (2013), different assesement approaches by an ecologocial footprint calculation and by the combination of AHP and PROMETHEE showed the same ranking for the options evaluated. However, it was remarked that defining weights was still influenced by the subjective opinion of the decision makers, even with the use of AHP (Herva & Roca, 2013).

This study has evaluated several different species of microalgae (*Chlorella protothecoides* and *Chlorella zofingiensis*), yeasts (*Cryptococcus albidus* and

Rhodotorula mucilaginosa), and fungi (*Aspergillus oryzae* and *Mucor plumbeus*) for microbial oil production using MCA. Firstly, microbial oil production by different strains was conducted with three different substrates (*i.e.*, glucose and xylose, as a model lignocellulosic hydrolysates, and glycerol). The data collected was used by the MCA approach to analyse factors and to select high ranking candidates using PROMETHEE-GAIA. The results from this study have shown that the MCA approach can be used for the selection of microorganisms for oil production that can be used as feedstock for biodiesel production.

2. Materials and Methods

2.1 Strains and media

Six microorganisms (of different origins) were selected for study based on the information obtained from the literature that are capable to cultivate oil. Two microalgal strains, *Chlorella protothecoides* (ATCC 30581) and *Chlorella zofingiensis* (ATCC 30412) were purchased from ATCC (USA). The composition of the basic medium used for microalgae strains was (per L): 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 25 mg $CaCl_2 \cdot 7H_2O$, 25 mg NaCl, 3 mg $FeSO_4 \cdot 7H_2O$, 0.01 mg vitamin B_1, and 1 mL A5 trace mineral solution at pH 6.8 (Bahadar & Bilal Khan, 2013). The A5 solution consisted of (per L) 2.86 g H_3BO_4, 2.5 g $MnSO_4 \cdot 7H_2O$, 22.2 mg $ZnSO_4 \cdot 7H_2O$, 7.9 mg $CuSO_4 \cdot 5H_2O$ and 2.1 mg $Na_2MoO_4 \cdot 2H_2O$ (Bahadar & Bilal Khan, 2013). Two yeast strains, *Cryptococcus albidus* (FRR no.: 2412) and *Rhodotorula mucilaginosa* (FRR no.: 2406) were purchased from FRR Culture Collection (Australia). The composition of the basic medium used for yeasts strains was (per L): 0.4 g $MgSO_4 \cdot 7H_2O$, 2 g KH_2PO_4, 3 mg $MnSO_4 \cdot H_2O$ and 0.1 mg $CuSO_4 \cdot 5H_2O$ at pH 5.5 (Yu et al., 2011). Two

fungal strains, *Aspegillus oryzae* (FRR no.: 1677) and *Mucor plumbeus* (FRR no.: 2412) were purchased from FRR Culture Collection (Australia). The composition of the basic medium used for fungal strains was (per L): 1 g KNO_3, 2.5 g KH_2PO_4, 10 mg $ZnSO_4 \cdot 7H_2O$, 2 mg $CuSO_4 \cdot 5H_2O$, 10 mg $MnSO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$ and 0.1 g $CaCl_2$ at pH 5.5 (Fu et al., 2010). Glucose, xylose and glycerol (30 g/L) were used as the carbon sources in the media supplemented with 4 g/L yeast extract. Cultures were conducted in triplicate in 500 mL Erlenmeyer flask containing 200 mL media placed on orbital shaking incubator (Ratex, Australia). Microalgal and yeast strains were cultivated with 20% (*v*/*v*) inoculums from their respective precultivation medium (4 days), at an orbital rate of 180 rpm with temperature maintained at 28 °C. The cultivation for microalgae strains was carried out in the dark. Fungal strains were cultivated with inoculum from 24 h of precultivation, at an orbital rate of 160 rpm with temperature maintained at 30 °C (Muniraj et al., 2013).

Microalgal and yeasts biomass were harvested by centrifugation at 6805 g for 7 min (Sorvall Biofuge Primo R, USA) (Xiong et al., 2008). Fungal biomass was harvested by vacuum filtration (Whatman 54 filter paper). The harvested biomass samples were washed three times (200 mL/wash) using Millipore water and freeze-dried to a constant weight.

2.2 Oil extraction

Oil was extracted from the biomass by Accelerated Solvent Extraction (ASE) technique using Dionex ASE 350 (Thermo Fisher Scientific Inc., USA). The samples for extraction were prepared by mixing dry biomass (~0.1 g) with 0.4 g of diatomaceous earth (Thermo Fisher Scientific, Inc., USA) and loaded into 11 mL cells. The extraction

conditions had been optimised and were as follows: temperature, 130 ℃; static time, 5 min; rinse volume, 25% of cell volume; purge time, 60 s; and using 4 static cycles. The solvent used was a mixture of chloroform:methanol in a ratio of 2:1 (*v*/*v*) (Mulbry et al., 2009). The extracted oil was collected in pre-weighed collection bottles. The solvents were evaporated under a stream of nitrogen. Unless otherwise specified, all results are reported on a dry weight (DW) basis.

2.3 Oil analyses

Sugars and glycerol concentrations were analysed using high-performance liquid chromatography (HPLC) by a Waters HPLC system equipped with a SP810 carbohydrate column (300 mm × 8.0 mm, Shodex, Japan) and a refractive index (RI) detector (Waters 410, US). The column temperature was 85 °C and the mobile phase was water, with a flow rate of 0.5 mL/min (Zhanying Zhang et al., 2013).

For the determination of fatty acids composition, fatty acid methyl esters (FAME) were prepared using the method described by Mulbry et al. (2009). FAME analysis was performed by gas chromatography-mass spectrometry (GC-MS) by Shimadzu GCMS-TQ8040 (Shimadzu Corporation, Japan) on a TG-WAXMS column (30 m long × 0.32 mm I.D. × 1 μm film thickness; Thermo Fisher Scientific, Inc., USA). The carrier gas was helium at a flow rate of 1.5 mL/min. A 10:1 split injection was used. The injection temperature was set at 230 °C, the MS ion source temperature at 220 °C and the MS interface temperature at 240 °C. The GC-MS method was carried out using the following temperature program: initial temperature at 40 °C, hold for 2 min, followed by 10 °C/min ramp to 230 °C and hold for 20 min. Mass spectrometry was performed using Q3 scan with an *m*/*z* 20-650 scanning range. Chromatograms and

mass spectra were evaluated using the GCMS solution software (Shimadzu Corporation, Japan). The retention times and mass spectra were identified using FAME mix (F.A.M.E. Mix, C8-C24; Sigma-Aldrich, Australia).

2.4 Multi-criteria analysis

2.4.1 Establishing criteria hierarchy

AHP and PROMETHEE-GAIA were used for MCA. PROMETHEE-GAIA was implemented using Visual PROMETHEE 1.4 Academic Edition. The stated goal of the MCA was to select the most suitable prospective microorganism(s) for oil production from lignocellulosic hydrolysates' model compounds, glucose and xylose. The alternative solutions were selected to be the six microorganisms studied which were *C. protothecoides, C. zofingiensis, C. albidus, R. mucilaginosa, A. oryzae* and *M. plumbeus*. Fig 1 shows the criteria hierarchy that was established from the key parameters reported in section 1. The quantitative criteria under Cluster 1 (*C1 – C6*) were evaluated based on the results of the experimental study on microbial cultivation on glucose, xylose and glycerol substrates. The criteria under Cluster 2 (*C7* and *C8*) were evaluated qualitatively.

2.4.2 Establishing criteria weights

AHP techniques were used to determine the relative weightings of each criterion (Herva & Roca, 2013; Macharis et al., 2004). This was based on hierarchy, priority setting and logical consistency (Macharis et al., 2004; Saaty, 2008). Relative priorities were given to each element through pairwise comparisons using Saaty's scale 1-9, whereby 1 indicates equal, 3 moderate, 5 strong, 7 very strong and 9 extreme

importance (Herva & Roca, 2013; Saaty, 2008). The scales of 2, 4, 6 and 8 were used for compromise values of importance (Saaty, 2008). The consistency of each pairwise comparison in this study was calculated, where the consistency falls within the range of the good consistency ratio (CR) proposed by Saaty (2008). There is the possibility of random judgement in assessing the priorities, if the consistency ratio is more than 10% (Saaty, 2008). The pairwise matrices for criteria groups belonging to each cluster and criteria of Cluster 1 are provided in Supplementary Table 1 and Table 2.

As shown in Fig. 1, Cluster 1 evaluates the relative capability of the alternative solutions for cultivation on the various carbon sources in order to achieve the goal. The priorities given to the carbon substrates are based on the capability of the respective microorganisms to grow and produce oil with the highest priority on glucose and the lowest priority on glycerol (Glucose (*G*) > Xylose (*X*) > Glycerol (*L*)). Higher priority is given to growth on glucose than xylose as lignocellulosic biomass generally consists of higher glucose than xylose. Glycerol was also included in the criteria as a potential substrate as glycerol is a by-product of biodiesel production and a potential fermentation substrate in an integrated biodiesel production system. The capability to grow on glycerol is assigned with a low weight as it only serves to provide a secondary benefit to the objective compared to the primary benefit resulting from growth on glucose and xylose.

The criteria assigned under Cluster 1, *C1* - *C6* were assessed with the priorities of *C1* > *C2* > *C3* > *C4* > *C5* > *C6*. The first two criteria, *C1* (oil concentration) and *C2* (oil content) were given the highest priority as they reflect the key economic advantage resulting from high concentration and yield of the desired product from each carbon substrate. Substrate consumption rate (*C3*) reflects the potential economic benefit of

lower capital and operating costs from reduced fermenter capacity. Oil yield (C4) reflects the efficiency with which the microorganism converts the substrate (which is an operating cost) to product (which is a revenue). Fatty acid profile (*C5*) evaluates the relative value of the oil for use in biodiesel production, and is calculated as the percentage of saturated and mono-unsaturated fatty acids in the oil produced. Oils with high levels of saturated and monounsaturated fatty acids are desirable for biodiesel application (Aransiola et al., 2014). Polyunsaturated fatty acids especially those with more than four double bonds are less preferred for biodiesel production due to the low oxidative stability of the biodiesel during storage (Chisti, 2007). However, microbial oils with high level of polyunsaturated fatty acids, such as linoleic acid (C18:2n6) and linolenic acid (C18:3n3) have the potential to be used in health products manufacturing (Huang et al., 2013). Oil productivity (*C6*) reflects average oil concentration per day of cultivation.

For the criteria belonging to Cluster 2 (*C7* and *C8*), the alternatives studied were categorised based on the classes of microorganisms as each alternative in the same class were assumed to share similar characteristics. For evaluating qualitative criteria, a 5-point scale was used (very good, good, average, bad, and very bad). Fungi were classified as very good for *C7* (Biomass harvesting cost) because fungal strains generally grow in pellet form. Pellet form is preferable for harvesting as the biomass can be harvested by simple sedimentation and filtration, whereas single cell biomass requires centrifugation or finer filtration techniques. Harvesting by sedimentation and filtration is a lower cost harvesting technique compared to harvesting via centrifugation (Chen et al., 2011). For criterion *C8* (Nutrient cost), yeasts were given the best ranking

as yeast species generally require fewer nutrients in the media compared to other microorganisms for oleaginous cultivation (Kitcha & Cheirsilp, 2011).

2.4.3 Ranking of alternatives

In PROMETHEE, the preference function converts the deviations between the evaluation of two alternatives for each criterion into a preference degree ranging from 0 to 1 (Behzadian et al., 2010). The preference functions used in this study are V-shape functions for quantitative criteria, and the usual function for qualitative criteria (Brans & Mareschal, 2005). V-shape function specifies values of preference threshold, p, which is the smallest deviation that is considered as sufficient to generate a full preference (Brans & Mareschal, 2005). The indifference threshold, q, is the largest deviation that is considered negligible by the decision maker and is equal to 0 in the V-shape function (Brans & Mareschal, 2005). The values of p in this study were determined using the built-in Preference Function Assistant in Visual PROMETHEE.

GAIA was used to further analyse and visualise the outcomes of the analysis. The following elements refer to results shown in the GAIA plane (Brans & Mareschal, 2005; Herva & Roca, 2013): (1) The criteria are represented by axes. Axes are oriented in approximately the same direction for criteria expressing similar preference and in opposite directions for conflicting criteria. Axes are oriented orthogonally for unrelated criteria. (2) Alternatives are represented by shapes. Alternatives with similar profiles are positioned close to each other. Alternatives with better performance on a given criterion are located in the direction of the corresponding criterion. (3) The weights of criteria are represented by the *pi* vector on the decision axis. The orientation of this axis shows which criteria are in accordance with PROMETHEE rankings and which are not.

3. Results and discussion

3.1 Biomass concentrations and carbon substrate consumptions on glucose, xylose and glycerol

Fig. 2 (a) shows the biomass concentrations of the six selected microorganisms growing on glucose, xylose and glycerol substrates. The yeast strain, *R. mucilaginosa* gave the highest biomass concentration of 16.79 g/L on glucose, while the other microorganisms had similar biomass concentrations on glucose ranging from 7.94 g/L to 9.81 g/L. One possible reason for the high biomass concentration of *R. mucilaginosa* is that the cultivation was not carried out in complete darkness. Biomass production from other species of *Rhodotorula, R. glutinis* was shown to be significantly enhanced from cultivation under light irradiation conditions (Zhiping Zhang et al., 2014). For cultivation on xylose, *R. mucilaginosa*, *A. oryzae* and *M. plumbeus* all resulted in high biomass concentrations of 10.78 g/L, 10.02 g/L and 9.32 g/L respectively. However, no significant biomass growth resulted from *C. protothecoides* and *C. zofingiensis* cultivation when xylose was used as the carbon source. These results are in agreement with a previous study that showed *Chlorella* species (*e.g.*, *C. vulgaris* and *C. sorokiniana*) were not able to assimilate xylose heterotrophically (Hawkins, 1999). Fungal strains *M. plumbeus* and *A. oryzae* also showed the highest biomass concentrations on glycerol (10.18 g/L and 9.49 g/L respectively). The results showed that both fungal strains, *M. plumbeus* and *A. oryzae,* and both yeasts strains, *R. mucilaginosa* and *C. albidus,* were able to grow on each of the three carbon sources studied. Interestingly, *M. plumbeus* and *A. oryzae* showed relatively consistent biomass concentrations on glucose, xylose and glycerol substrates.

The results of substrates consumption by the six microorganisms studied over 168 h of cultivation are shown in Fig. 3. All six microorganisms were shown to consume glucose more rapidly than xylose and glycerol. Generally, glucose is more preferable than xylose as a fermentation substrate as assimilation of xylose requires specific metabolic pathways (Zheng et al., 2012). Glucose was shown to be completely consumed by fungal strains *A. oryzae* and *M. plumbeus* within only 48 h to 72 h of cultivation. Yeast strain *R. mucilaginosa* and microalgae strain *C. protothecoides* consumed glucose completely by the end of the cultivation period. Xylose was completely consumed in the media by *A. oryzae* in 96 h, whereas it took 144 h for *M. plumbeus* and *R. mucilaginosa* to consume xylose completely. No significant consumption of xylose was evident for either of the microalgae strains. All microorganisms consumed glycerol at a slower rate than glucose and xylose. Consumption of glycerol was again the fastest for the fungal species *A. oryzae* and *M. plumbeus*.

The two fungal strains, *A. oryzae* and *M. plumbeus,* demonstrated the highest consumption rates on glucose, xylose and glycerol. It is known that upon depletion of the carbon source, there exists the possibility of lipid turnover, in which storage lipids are metabolised resulting in a reduction in lipid content (Fakas et al., 2007). In this study, the lipid content was not monitored at each time point as the work focused on the development of MCA method for screening and selection of optimal oil producing microorganisms. It is noted, however, that the peak oil content for microorganisms with rapid substrate consumption may have been higher than the results show.

3.2 Microbial oil production from different carbon substrates

Fig. 2 (b) shows the results of the oil contents of the strains on glucose, xylose and glycerol substrates after 168 h cultivation. *C. protothecoides* cultivation on glucose showed the highest oil content of 35.44% (*w/w*), followed by *A. oryzae* (26.86%), *M. plumbeus* (26.17%), *C. zofingiensis* (24.7%), *R. mucilaginosa* (21.55%) and *C. albidus* (19.46%). It has been demonstrated in previous studies that *C. protothecoides* is an excellent oil producer on glucose with up to 58% oil content obtained from batch cultivation in a 5 L bioreactor for 140 h (Xiong et al., 2008). The highest oil content on xylose was achieved by *M. plumbeus*, which was 23.83%, followed by *A. oryzae, C. albidus* and *R. mucilaginosa* (oil contents of 20.65%, 18.30% and 14.41% respectively). As there was almost no growth of *Chlorella* strains on xylose, the oil content was not measured. The highest oil contents on glycerol were achieved by *M. plumbeus*, *A. oryzae*, and *C. albidus*, which were all around 26% (27.39%, 25.79% and 26.41% respectively). Lower oil contents on glycerol substrates were shown by *R. mucilaginosa*, *C. protothecoides* and *C. zofingiensis*.

Fig. 2 (b) also shows that *A. oryzae* and *M. plumbeus* had consistent oil contents with varying carbon sources. Although the two fungal strains had ~8-9% lower final oil contents than *C. protothecoides*, these strains grew much faster and are likely to result in comparable or higher oil productivity. Yeast strain *R. mucilaginosa* produced the highest biomass concentration while still producing similar oil contents to most of the other strains.

Fig. 2 (c) shows oil concentrations for the six microorganisms growing on glucose, xylose and glycerol. The cultivation of *R. mucilaginosa* on glucose resulted in

the highest oil concentration of 3.61 g/L primarily as a result of the very high biomass concentration compared to the other species. *C. zofingiensis* and the two fungal strains had similar oil concentrations on glucose. *M. plumbeus* showed the highest oil concentration on xylose and glycerol (2.21 g/L and 2.78 g/L respectively), followed by *A. oryzae* (2.07 g/L and 2.45 g/L respectively). Fig. 2 (c) also shows the consistency in the oil concentrations achieved by the two fungal strains across all three carbon sources compared to the other species which tended to be more variable with varying carbon substrates.

3.3 Fatty acids profiles

The results of the fatty acid compositions of the six microorganisms growing on glucose, xylose and glycerol are presented in Table 1. The major fatty acids identified were palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2). Oleic acid was the predominant fatty acid in most cases which is in accordance with previous studies (Meng et al., 2009). Variations were observed for the cultivation of *C. albidus* on glucose and xylose substrates, with linoleic acid as predominant fatty acid while palmitic acid was the predominant fatty acid with *C. zofingiensis* on glycerol. The reasons for high accumulation of palmitic acid by *C. zofingiensis* on glycerol are unknown as this is the first study to cultivate *C. zofingiensis* on glycerol. Nevertheless, this *C. zofingiensis* may have similar pathways to metabolise glycerol to *Chlorella saccharophila* reported previously, which produced palmitic acid as predominant fatty acid on glycerol but oleic acid on glucose (Isleten-Hosoglu et al., 2012; Singh et al., 2013). The composition of oleic acid was decreasing and palmitic acid was increasing with increasing ratio of glycerol mixed with glucose substrate (Isleten-Hosoglu et al., 2012).

3.4 Preference ranking

Based solely on the oil concentration results above, it could be concluded that, of the microorganisms assessed, *R. mucilaginosa* and *C. protothecoides* were the most prospective microorganisms for microbial oil production from glucose. On the other hand, *A. oryzae* and *M. plumbeus* appeared to be the most prospective for oil production from xylose. *M. plumbeus* had the lowest polyunsaturated fatty acid content when grown on glucose and hence potentially produced better oil for biodiesel production but had the highest polyunsaturated fatty acid when grown on glycerol. Furthermore, these initial conclusions ignore the impact of other aspects that impact on production cost including harvesting and nutrition costs. Therefore, PROMETHEE-GAIA was used to systematically assess each alternative based on the criteria shown above.

Fig. 4 (a) shows the results of the PROMETHEE I partial rankings for the six microorganisms studied. In PROMETHEE I, the presence of crossed tie lines indicate that the alternatives are not comparable using this technique. For instance, *M. plumbeus* is not comparable to *A. oryzae* because *M. plumbeus* obtained a higher *Phi-* (*negative preference flow*), and a lower *Phi+* (*positive preference flow*) compared to *A. oryzae*.

Fig. 4 (b) also shows the results of the PROMETHEE II complete rankings for the six microorganisms studied. The only microorganisms that obtained positive *Phi* scores were *A. oryzae*, *M. plumbeus* and *R. mucilaginosa* with the two fungal species *A. oryzae* and *M. plumbeus* being the most preferred options with almost equivalent *Phi* scores. As a result, based on the criteria selected and the experimental results, these three microorganisms (*A. oryzae*, *M. plumbeus* and *R. mucilaginosa)* were predicted to

be more preferred for oil production from the lignocellulosic hydrolysates model compounds, and glycerol than the other microorganisms.

The GAIA plane from the analysis is shown in Fig. 5 and has a quality level of 80.5% which is reliable as it is above 70% quality significance level. The *pi* decision axis is aligned in the direction of the fungal strains *A. oryzae* and *M. plumbeus*, which shows that these alternatives are preferred which is in agreement with the PROMETHEE II ranking.

In the GAIA plane, the criteria vectors that lie in the same direction as the decision vector reflect the influence that these criteria have on the decision. Fig. 5 shows that the substrate consumption rate (*C3*) and fatty acid profiles (*C5*) for all of the substrates express a positive preference on the decision.

Sensitivity analysis was carried out on the selected preference function and criteria weights. By substituting the V-shape preference function with linear function for all quantitative criteria without changing the preference threshold, (*p*), the PROMETHEE II ranking remains the same. The sensitivity of the criteria weights to the results are analysed based on weight stability intervals (Table 2). Weight stability intervals are the limits where any variation in weight within the intervals will not change the ranking of PROMETHEE II, given that there is no change to the relative weights of other criteria (Herva & Roca, 2013). Most of the criteria exhibited broad weight stability intervals which show that the analysis is robust.

The three highest ranking alternatives, *A. oryzae*, *M. plumbeus* and *R. mucilaginosa* were further analysed using GAIA Web to determine the influence of individual criteria on the preference result (Fig. 6). GAIA Web shows a graphical

representation of the unicriterion net flow scores for the selected alternative. The criteria axes in GAIA Web are positioned with the same orientation as in the GAIA plane, where criteria with similar preferences are located close to each other. The GAIA Web shows the key criteria with the radial distance indicating unicriterion net flows with -1 value at the centre of the web and +1 on the outer circle.

Fig. 6 (a) shows that *R. mucilaginosa* performed strongly for the criteria of oil concentration, oil yield and substrate consumption rate on glucose but the criteria of oil content, fatty acid profile and oil productivity on glycerol were weak. Oil concentration, oil content, and substrate consumption rate on xylose and glycerol were all weak. On the other hand, *A. oryzae* shows very good preference for oil concentration, oil content, and fatty acid profiles on xylose and glycerol and oil content and fatty acid profile on glucose. In fact it is noted that *A. oryzae* showed good preference results across most criteria with the exception of oil concentration and oil yield on glucose, and productivity and oil yield on glycerol. The fungal strain *M. plumbeus* showed very good preferences for most of the criteria on glucose, xylose and glycerol with the exception of oil concentration and oil yield on glucose, and productivity on glycerol.

The incomparability between *M. plumbeus* with *A. oryzae* in PROMETHEE I can be assessed through the GAIA Webs. A comparison of the GAIA Webs between these two species shows different strengths in preference between these fungal strains for criteria such as fatty acid profiles but the incomparability is not highly significant. The GAIA Webs confirmed the results obtained from the GAIA plane reflecting that *A. oryzae* and *M. plumbeus* showed good preference for most of the criteria specified.

The preferred alternatives for oil production for biodiesel production from highest to lowest were established as follows: (1) *A. oryzae*; (2) *M. plumbeus*; (3) *R. mucilaginosa*; (4) *C. protothecoides*; (5) *C. albidus* and (6) *C. zofingiensis*. The microorganisms with positive *Phi* scores (*A. oryzae*, *M. plumbeus* and *R. mucilaginosa*) were selected as the most prospective species and further analysed using unicriterion net flow analysis in GAIA Webs. The variations in positive preferences across these three microorganisms were confirmed by PROMETHEE I, the GAIA plane and also the GAIA Webs. Therefore, fungal strains *A. oryzae, M. plumbeus* and yeast strain *R. mucilaginosa* have potential for industrial oil production for biodiesel applications.

The MCA proposed can be improved for ranking and selecting the best microorganism for oil production from a specific type of hydrolysates, whereby the priority for carbon substrates can be adjusted accordingly. MCA for oil production from hydrolysates of liquid fraction of pretreated lignocellulosic materials may include microorganisms' tolerance to inhibitors such as furfural and 5-hydroxymethylfurfural, as one of the criteria for ranking and selecting the best microorganism.

4. Conclusion

In this study, a MCA approach was used to evaluate the performance of oil production with different microorganisms. The MCA technique using AHP and PROMETHEE-GAIA showed that the only microorganisms with positive *Phi* scores were *A. oryzae*, *M. plumbeus* and *R. mucilaginosa*. Further GAIA analyses showed that the fungal strains *A. oryzae* and *M. plumbeus* provided superior performance across a wide range of criteria including growth on glucose and xylose substrates. Overall, *A.*

oryzae, M. plumbeus and *R. mucilaginosa* showed promise for biodiesel production using the lignocellulose hydrolysates model compounds, glucose and xylose.

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Figure Legends

Fig. 1. Criteria hierarchy for the evaluation of microorganisms for microbial oil production. Cluster 1 criteria were evaluated based on cultivation results on glucose (*G)*, xylose (*X)* and glycerol (*L).*

Fig. 2. (a) Biomass concentration, (b) oil content and (c) oil concentrations for growth of six microorganisms on glucose, xylose and glycerol.

Fig. 3. Consumption of (a) glucose, (b) xylose and (c) glycerol over 168 h of cultivation.

Fig. 4. (a) PROMETHEE I partial ranking of alternatives and (b) PROMETHEE II complete ranking where *RM* denotes *R. mucilaginosa*, *AO A. oryzae*, *MP M. plumbeus*, *CP C. protothecoides*, *CA C. albidus* and *CZ C. zofingiensis*.

Fig. 5. GAIA plane at (a) 100% zoom and (b) 400% zoom without the alternatives. The alternatives are denote as *RM* for *R. mucilaginosa, AO* for *A. oryzae, MP* for *M. plumbeus, CP* for *C. protothecoides, CA* for *C. albidus* and *CZ* for *C. zofingiensis*. The criteria are denotes as *C1-G* to *C6-G* for criteria of Group 1.1 (Glucose), *C1-X* to *C6-X* for criteria of Group 1.2 (Xylose) and *C1-L* to *C6-L* for criteria of Group 1.3 (Glycerol). Some criteria are not visible due to overlapping such as *C1-X* by *C1-L*, *C5-X* and *C3-X* by *C3-L*, *C5-G* by *C7*, *C2-X* and *C6-X* by *C2-L*.

Fig. 6. GAIA Webs for top three alternatives from PROMETHEE which are (a) *R. mucilaginosa*, (b) *A. oryzae* and (c) *M. plumbeus*. Criterion *C2-X* is not visible due to overlapping by *C6-X*.

Fig. 1

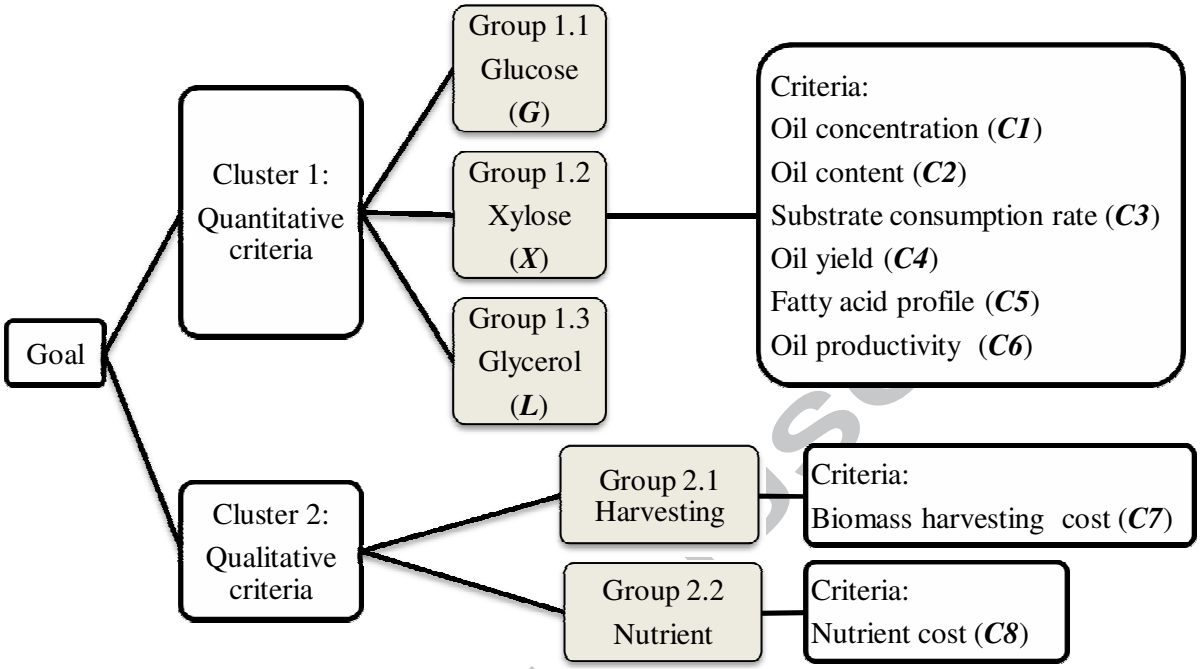

Fig. 2.

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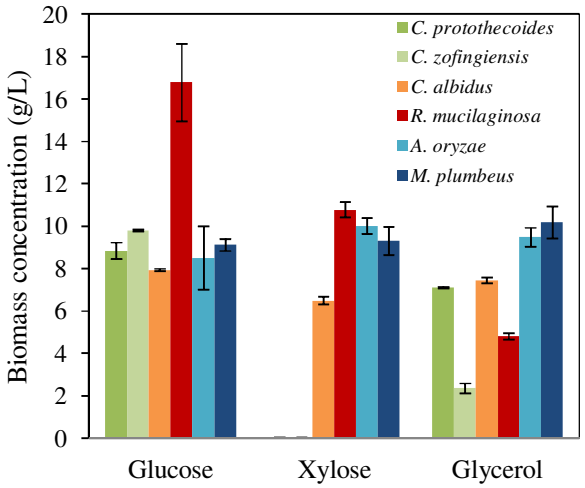

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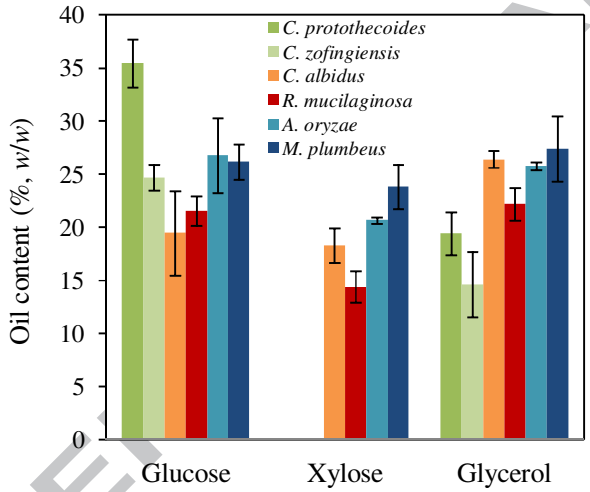

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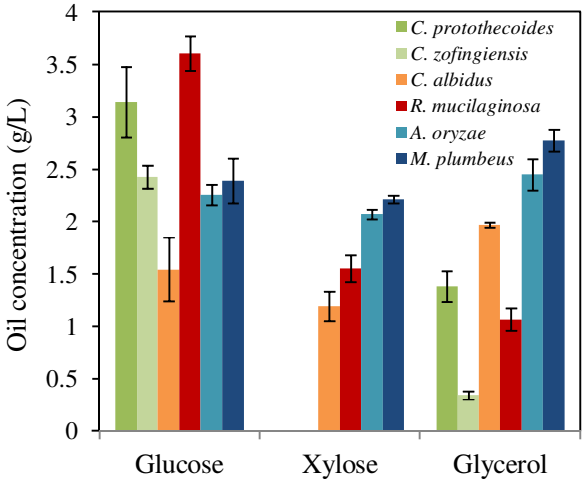

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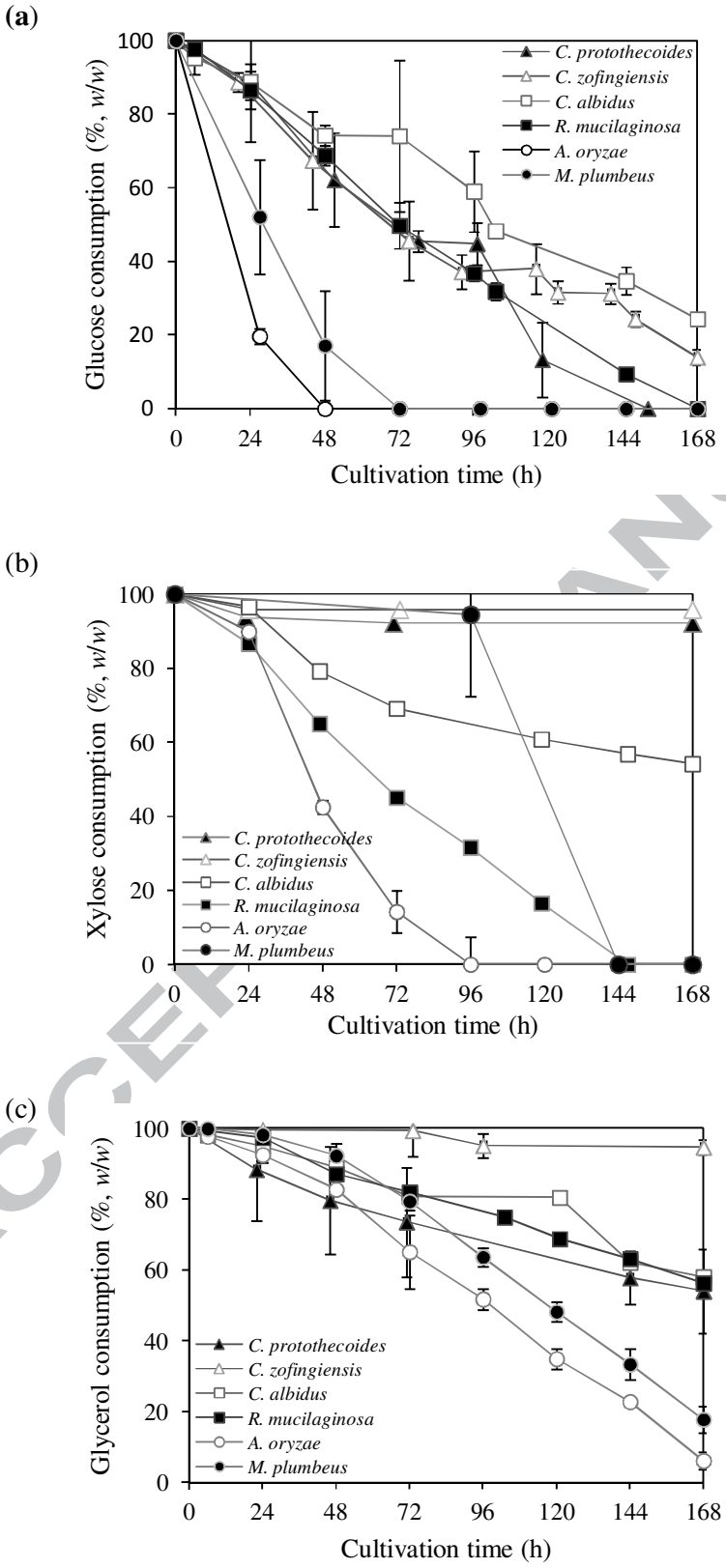

Fig. 4.

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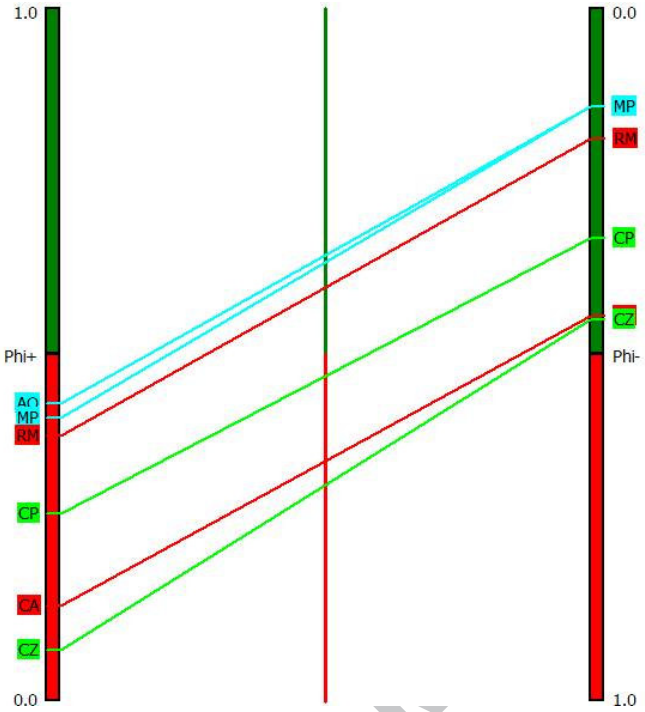

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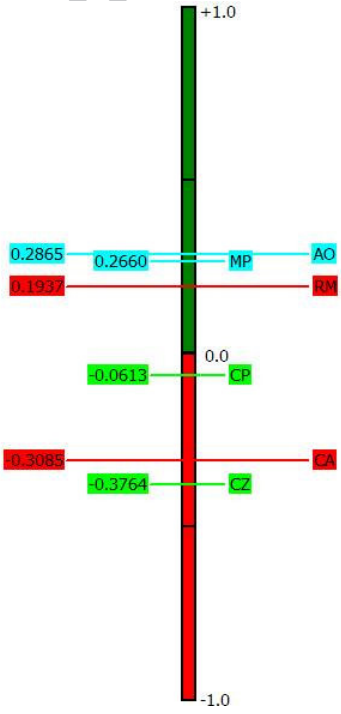
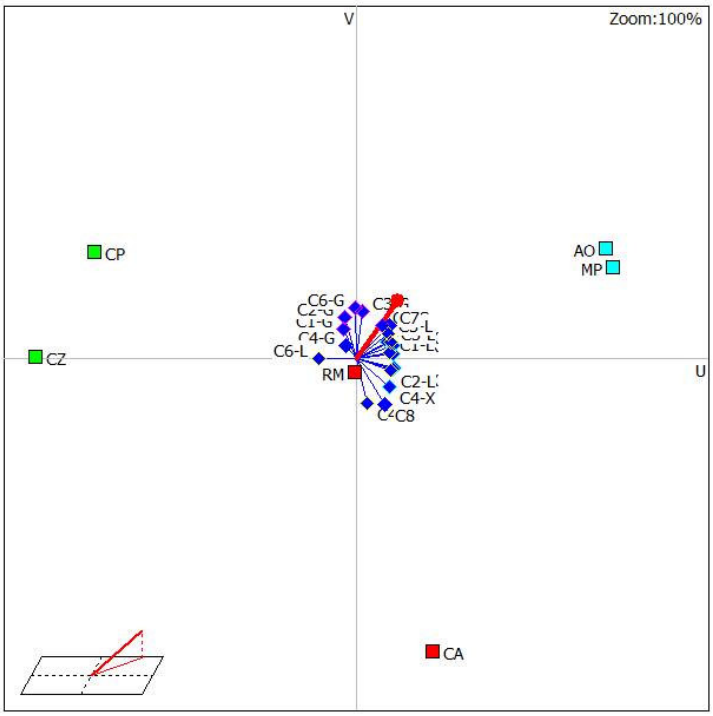


Fig. 5.

(a)



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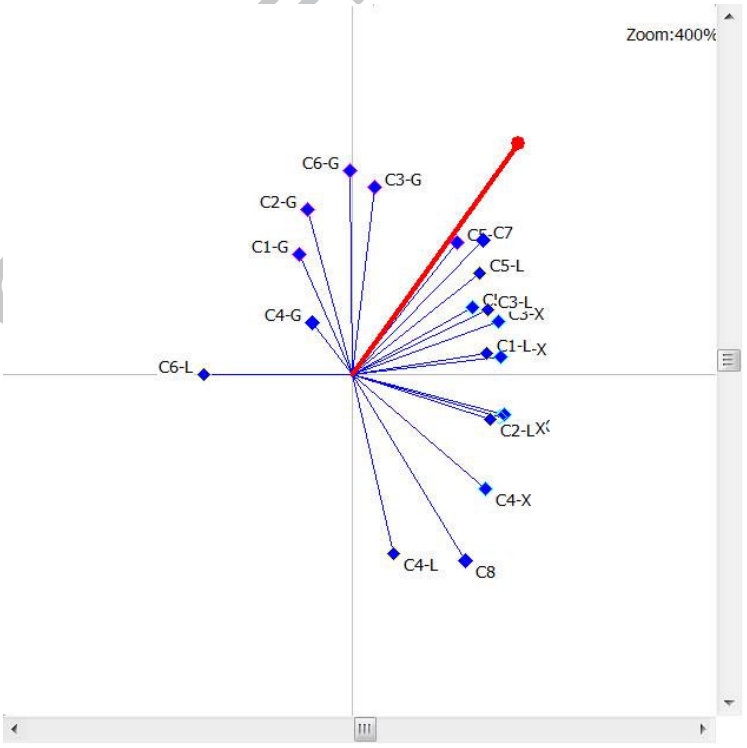
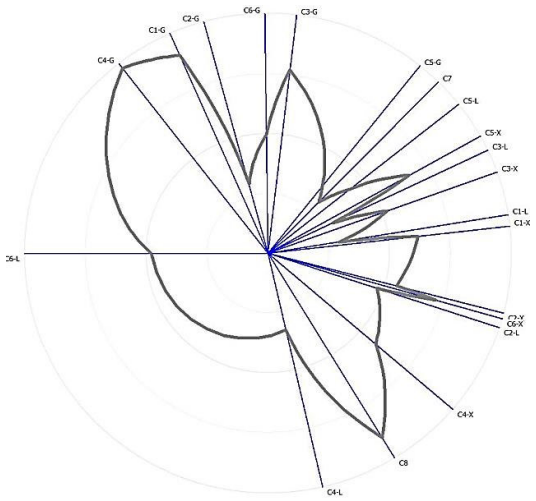


Fig. 6.

(a)



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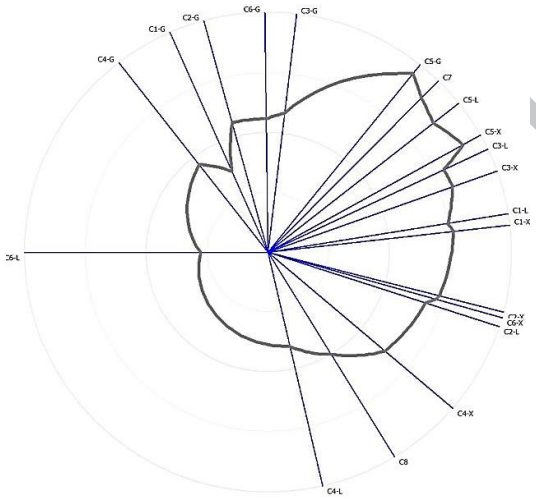

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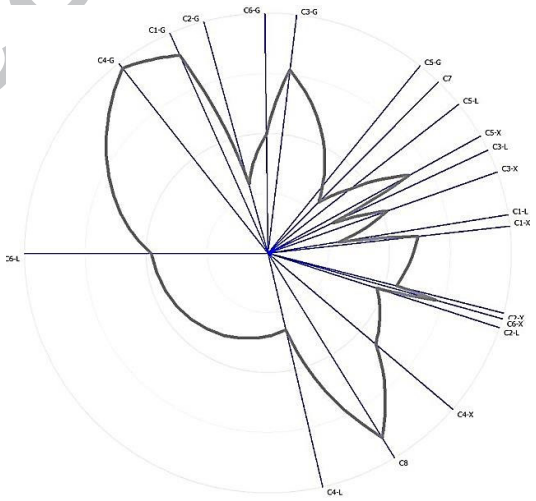

Table 1. Fatty acid compositions of oil extracted from six different microorganisms grown on various carbon substrates.

Microorganisms	Relative abundance of total fatty acids (%, *w*/*w*)								SFA[a]	MUFA[a]	PUFA[a]
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C20:0			
Glucose medium											
C. protothecoides	6.3 (±2.0)	23.8 (±1.5)	3.8 (±0.7)	6.1 (±0.7)	42.8 (±2.6)	5.1 (±3.0)	6.8 (±2.4)	-	38.2	49.2	12.6
C. zofingiensis	7.2 (±1.1)	22.4 (±1.9)	7.8 (±2.0)	6.9 (±3.0)	42.2 (±2.7)	6.5 (±9.8)	4.0 (±3.8)	-	37.6	51.5	10.8
C. albidus	1.5 (±0.6)	22.7 (±0.1)	1.7 (±0.4)	4.1 (±1.3)	34.1 (±2.5)	35.8 (±2.2)	-	-	28.4	35.8	35.8
R. mucilaginosa	2.7 (±0.7)	18.9 (±3.7)	1.7 (±0.3)	6.9 (±2.2)	54.2 (±6.5)	12.6 (±8.4)	2.9 (±0.9)	-	28.6	55.9	15.5
A. oryzae	4.5 (±2.8)	25.5 (±4.4)	3.4 (±0.4)	15.6 (±6.0)	34.9 (±2.9)	9.8 (±8.3)	3.1 (±0.5)	1.1 (±0.7)	47.7	39.1	13.2
M. plumbeus	2.0 (±0.8)	28.8 (±0.8)	2.5 (±0.5)	22.1 (±1.7)	37.4 (±0.6)	2.8 (±2.1)	1.1 (±0.7)	1.6 (±0.3)	55.4	40.6	4.00
Xylose medium											
C. albidus	-	29.5 (±2.0)	-	13.4 (±1.8)	23.4 (±2.2)	33.7 (±0.3)	-	-	42.9	23.4	33.7
R. mucilaginosa	1.8 (±0.2)	20.3 (±1.7)	1.11 (±0.3)	6.1 (±0.8)	49.2 (±3.1)	20.1 (±5.3)	1.3 (±1.2)	-	28. 3	50.4	21.4
A. oryzae	0.8 (±0.1)	20.5 (±1.9)	1.7 (±0.3)	16.4 (±0.7)	37.5 (±0.9)	21.1 (±3.2)	-	1.5 (±0.1)	39.4	39.4	21.2
M. plumbeus	1.3 (±0.6)	20.5 (±4.0)	1.8 (±0.6)	19.0 (±1.7)	33.8 (±3.1)	21.1 (±9.0)	1.0 (±0.8)	1.5 (±0.3)	42.2	35.7	22.1
Glycerol medium											
C. protothecoides	10.3 (±1.2)	26.6 (±0.2)	4.4 (±1.5)	6.5 (±0.5)	35.9 (±4.3)	7.0 (±9.4)	2.1 (±1.8)	-	46.8	43.4	9.9
C. zofingiensis	17.7 (±4.2)	56.4 (±3.2)	-	8.2 (±2.0)	10.4 (±6.4)	7.3 (±2.4)	-	-	82.2	10.4	7.3
C. albidus	1.5 (±0.1)	24.4 (±1.1)	1.9 (±0.3)	5.5 (±2.2)	42.4 (±2.3)	24.3 (±0.6)	-	-	31.4	44.3	24.3
R. mucilaginosa	4.6 (±1.2)	14.7 (±1.3)	1.6 (±0.2)	9.1 (±1.9)	47.6 (±3.6)	6.6 (±2.5)	15.7 (±3.2)	-	28.4	49.3	22.4
A. oryzae	0.9 (±0.5)	14.2 (±0.9)	1.9 (±0.5)	16.9 (±0.8)	34.4 (±1.1)	29.3 (±1.3)	0.5 (±0.0)	1.8 (±0.3)	33.9	36.4	29.8
M. plumbeus	0.5 (±0.7)	14.1 (±0.0)	2.8 (±1.1)	14.3 (±0.2)	30.9 (±2.4)	35.6 (±2.1)	0.5 (±0.2)	1.3 (±0.2)	30.2	33.7	36.1

[a] SFA means saturated fatty acids, MUFA means monounsaturated fatty acids and PUFA means polyunsaturated fatty acids.

Table 2. Weight stability intervals for criteria with relative weight >5%.

Criteria		Weight (%)	Weight stability intervals
C1-G	Oil concentration on glucose	22.50	[2.70 - 28.02]
C2-G	Oil content on glucose	11.22	[0 - 22.15]
C3-G	Consumption rates on glucose	7.41	[3.40 - 71.58]
C1-X	Oil concentration on xylose	11.95	[2.51 - 34.65]
C2-X	Oil content on xylose	5.96	[0 - 19.23]

HIGHLIGHTS

- First MCA to rank microorganisms from various classes for biodiesel production.
- Oil production by various microorganisms was studied on different carbon sources.
- Major fatty acids: Palmitic, stearic, oleic and linoleic acid.
- *A. oryzae*, *M. plumbeus* and *R. mucilaginosa* scored positive *Phi* from PROMETHEE II.
- Fungi strains showed superior performance across majority of criteria from GAIA Webs.